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Symbiotic Human Gut Bacteria with Variable Metabolic Priorities for Host Mucosal Glycans

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ABSTRACT Many symbiotic gut bacteria possess the ability to degrade multiple polysaccharides, thereby providing nutritional advantages to their hosts. Like microorganisms adapted to other complex nutrient environments, gut symbionts give different metabolic priorities to substrates present in mixtures. We investigated the responses of *Bacteroides thetaiotaomicron*, a common human intestinal bacterium that metabolizes more than a dozen different polysaccharides, including the O-linked glycans that are abundant in secreted mucin. Experiments in which mucin glycans were presented simultaneously with other carbohydrates show that degradation of these host carbohydrates is consistently repressed in the presence of alternative substrates, even by *B. thetaiotaomicron* previously acclimated to growth in pure mucin glycans. Experiments with media containing systematically varied carbohydrate cues and genetic mutants reveal that transcriptional repression of genes involved in mucin glycan metabolism is imposed by simple sugars and, in one example that was tested, is mediated through a small intergenic region in a transcript-autonomous fashion. Repression of mucin glycan-responsive gene clusters in two other human gut bacteria, *Bacteroides massiliensis* and *Bacteroides fragilis*, exhibited variable and sometimes reciprocal responses compared to those of *B. thetaiotaomicron*, revealing that these symbionts vary in their preference for mucin glycans and that these differences occur at the level of controlling individual gene clusters. Our results reveal that sensing and metabolic triaging of glycans are complex processes that vary among species, underscoring the idea that these phenomena are likely to be hidden drivers of microbiota community dynamics and may dictate which microorganisms preferentially commit to various niches in a constantly changing nutritional environment.

IMPORTANCE Human intestinal microorganisms impact many aspects of health and disease, including digestion and the propensity to develop disorders such as inflammation and colon cancer. Complex carbohydrates are a major component of the intestinal habitat, and numerous species have evolved and refined strategies to compete for these coveted nutrients. Our findings reveal that individual bacteria exhibit different preferences for carbohydrates emanating from host diet and mucosal secretions and that some of these prioritization strategies are opposite to one another. Thus, we reveal new aspects of how individual bacteria, some with otherwise similar metabolic potential, partition to “preferred niches” in the complex gut ecosystem, which has important and immediate implications for understanding and predicting the behavioral dynamics of this community.

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Bacteria in the human colon have developed several strategies to forage the complex carbohydrates (polysaccharides or glycans) present in dietary fiber. With the exception of starch, members of this diverse group of nutrients transit the proximal intestine undigested due to a lack of appropriate human enzymes. Residual dietary glycans therefore require the activity of symbiotic bacteria in the distal gut first for hydrolysis (1) and then for fermentation into host-absorbable short-chain fatty acids, which provide a significant contribution to our dietary energy harvest (2). Human intestinal cells also secrete endogenous glycans, largely as mucin glycoproteins and epithelial glycocalyx, which contribute to a protective barrier for host tissue. Most glycans associated with intestinal mucins are O-linked glycans (herein referred to as O-glycans) to the side chains of serine and threonine

residues in mucin polypeptides. Encompassing $\sim 10^2$ different linear and branched structures, the O-glycans that are attached to mucins are remarkably diverse and also distinct from dietary fiber glycans (3, 4). Thus, to metabolize O-glycans, gut bacteria require highly diversified sensory systems coupled to expression of dozens of appropriate enzymes (5, 6).

A number of species from the dominant human gut bacterial phyla (*Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Verrucomicrobia*) have been associated with degradation of mucus and/or its attached O-glycans, while other species in these phyla lack this behavior (7–11). Several experiments with simplified or transplanted gut microbial communities assembled in gnotobiotic mice have implied that the ability to forage host-derived glycans provides a metabolic alternative when dietary glycans wane or are

absent or when microorganisms are forced into competition by the addition of additional microbial species (12–17).

Detrimental host effects associated with disrupted mucin glycosylation and bacterial degradation of host glycan structures are beginning to emerge as well. Studies using mouse models that lack normal mucin expression or glycosylation have observed spontaneous colonic inflammation or increased susceptibility to inflammatory agents, including individual bacteria (18–21), raising the possibility that microbe-catalyzed alterations to mucus could also be harmful. This notion is supported by findings in humans with ulcerative colitis, which show that *O*-glycosylation patterns are simpler in people with active disease versus the same patients during remission (22). Symbionts such as *Bacteroides thetaiotaomicron* can liberate sialic acid from host glycoconjugates, providing a sugar that it cannot use itself to pathogens such as *Salmonella enterica* and *Clostridium difficile* (23). Finally, a recent study involving mice that are genetically susceptible to acute colonic inflammation that can be induced by *B. thetaiotaomicron* or other commensal *Bacteroidetes* demonstrated that genetic disruption of bacterial sulfatase production blocks the progression of disease (24). The precise, disease-causing target(s) of *B. thetaiotaomicron* sulfatases has not yet been identified. However, the observation that most of the 28 different predicted sulfatase-encoding genes in the colitogenic *B. thetaiotaomicron* type strain (25) are contained in gene clusters that are responsive to host mucin *O*-glycans or glycosaminoglycans (6, 24) suggests that bacterial interactions with the sulfated carbohydrates abundant in colonic mucus or the glycocalyx are a critical step.

B. thetaiotaomicron and several other mucin-degrading *Bacteroides* species possess the ability to catabolize other carbohydrates in addition to *O*-glycans. To date, essentially all of these metabolic abilities that have been described are mediated by tightly regulated expression of corresponding polysaccharide utilization loci (PULs) that encode specific degradative machinery for the particular substrate(s) present (7). We have previously shown that when *B. thetaiotaomicron* is grown in a mixture containing a dozen different glycans, it represses expression of genes involved in *O*-glycan utilization until the latest stages of growth when most or all of the other substrates have been depleted (26). Here, we investigate the ability of individual glycans to modulate *O*-glycan metabolism in *B. thetaiotaomicron* and other gut species and provide molecular genetic insight into the basis of this phenomenon. We identify two species, *Bacteroides massiliensis* and *Bacteroides fragilis* that exhibit different, often reciprocal, *O*-glycan preferences compared to those of *B. thetaiotaomicron*. Given the likelihood that such metabolic variations extend to other nutrients, the phenomena described here represent an underappreciated and perhaps pivotal driver of dynamics in the diet-microbiota-host relationship.

RESULTS

***O*-linked glycans are given low metabolic priority by *B. thetaiotaomicron*.** The metabolic preferences and corresponding regulatory mechanisms exhibited by symbiotic bacteria in the human colon remain largely unexplored. We previously found that *B. thetaiotaomicron* preferentially metabolizes glycosaminoglycans prior to *O*-glycans when they are present together in a mixture (6). To test the hypothesis that other glycans repress *O*-glycan use, we grew *B. thetaiotaomicron* in pairwise mixtures of porcine mucin *O*-glycans (PMOG) and several other carbohy-

drates (Fig. 1). If either substrate was prioritized, we anticipated “diauxic” growth patterns, indicating metabolic shifts between the most- and least-preferred nutrients. In addition, we harvested bacterial RNA at “early” and “late” points in growth and compared glycan-specific PUL expression by quantitative PCR (qPCR) on reverse-transcribed RNA.

Data for a plant cell wall pectin (homogalacturonan), a bacterial β 2,6-linked fructan capsule (levan), and an animal tissue glycosaminoglycan (chondroitin sulfate) are shown in Fig. 1A to H. Consistent with our hypothesis, we observed diauxic growth in which the early growth phase was prolonged by increasing homogalacturonan, levan, or chondroitin sulfate (Fig. 1A, D, and G). Gene expression comparisons further support repression of *O*-glycan metabolism with high expression of sentinel *susC*-like genes from the homogalacturonan, levan, and chondroitin sulfate PULs early and reciprocal patterns for nearly all of the seven different *O*-glycan-responsive PULs that were tested (Fig. 1B, E, and H; see Fig. S1I in the supplemental material). In addition, *B. thetaiotaomicron* cells that were pregrown in PMOG abruptly repressed *O*-glycan PULs when homogalacturonan, levan, or a mixture of all glycans used was introduced (Fig. 1C and F; Fig. S2A).

Results similar to those with homogalacturonan, levan, and chondroitin sulfate were observed with nearly all other glycans tested (see Fig. S1A to S1H in the supplemental material). In aggregate, gene expression trends for glycans tested against PMOG revealed significant overall repression of *O*-glycan genes, underscoring that utilization of this host-derived nutrient is low in *B. thetaiotaomicron*’s metabolic hierarchy (Fig. 1I). Although *B. thetaiotaomicron* growth rates on PMOG are among the lowest compared to other glycans (Fig. 1J; see Fig. S3 for schematics of all glycans used), strong repression of *O*-glycan PULs in mixtures with levan or heparin, which both had similarly low growth rates, argues that growth rate is insufficient to account for this trend. Only dextran failed to exhibit significant expression changes between early and late growth (blue data points in Fig. 1I), although it still elicited a diauxic growth profile (Fig. S1C).

To determine whether the signals that induce repression of *O*-glycan PULs originate from the transcriptional sensor/regulators that activate expression of their cognate PULs in response to the alternative glycans that were presented with PMOG, as was reported in one previous study (27), or their component simple sugars, we performed experiments with the monosaccharides fructose and galactose. Fructose is the sole component of levan and is sufficient to trigger expression of the single levan PUL (28). Galactose is abundant in both pectic galactan and PMOG (6). The presence of either monosaccharide was sufficient to repress expression of *O*-glycan PULs (Fig. 1K and L). The observation that galactose decreases expression of *O*-glycan PULs reveals that a monosaccharide, in sufficient quantity, is capable of suppressing utilization of another polymer in which it is also contained. Moreover, activation of the repressing glycan’s cognate PUL, via its associated regulator, is not required because galactose does not induce expression of the pectic galactan PUL (Fig. 1L). Additional monosaccharide experiments further support the idea that simple sugars mediate repression, albeit with notable variation with respect to sugar identity and timing (see Fig. S2B to S2L in the supplemental material).

Finally, we grew *B. thetaiotaomicron* on mixtures containing human milk oligosaccharides (HMOs), glycans with structural

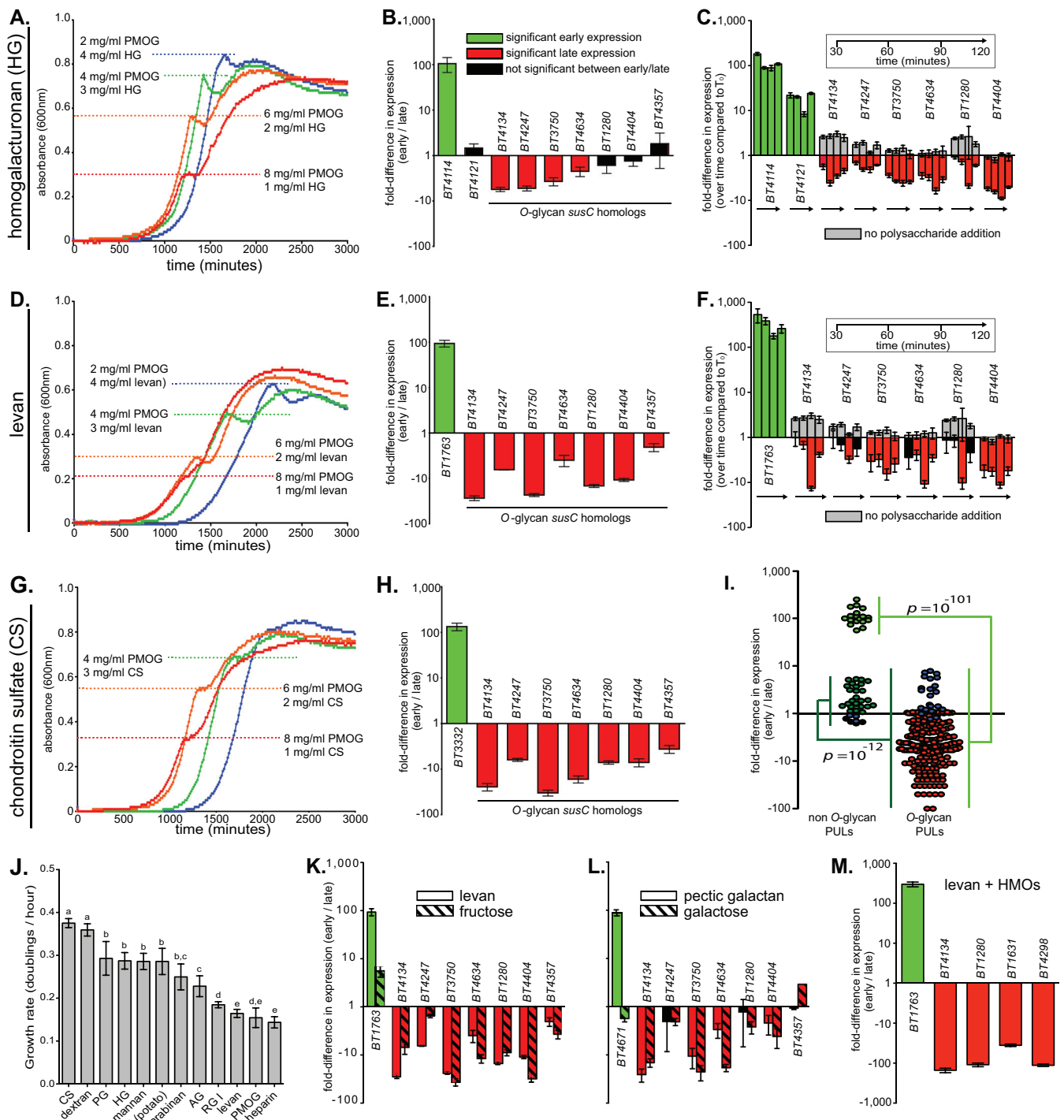


FIG 1 *B. thetaiotaomicron* deprioritizes O-glycan metabolism in the presence of competing glycans and monosaccharides. (A) *B. thetaiotaomicron* growth curves in mixtures of homogalacturonan (HG) and PMOG. The dashed horizontal lines indicate the pause in exponential growth that occurs as a function of changing glycan mix concentrations. (B) Relative expression differences between early and late growth phases for *B. thetaiotaomicron* grown in a mixture of HG and PMOG (1.5 mg/ml and 7 mg/ml, respectively). Early and late samples were harvested before and after the pause, respectively. A total of seven previously identified O-glycan-responsive PULs (6) were monitored for changes in expression based on the criteria that they were induced more than 8.5-fold in pure PMOG and are not part of recombinational shufflons that might obfuscate temporal expression. PULs with statistically significant ($P \leq 0.05$ by one-tailed Student's *t* test) increases in expression during early growth, PULs with statistically significant increases in later growth, and PULs with no significant changes in early versus late growth are shown. (C) A "spike-in" experiment in which *B. thetaiotaomicron* was grown to mid-exponential phase on 10 mg/ml PMOG alone and then HG (final concentration of 2.5 mg/ml) was abruptly introduced. Bacterial samples were harvested at 30-, 60-, 90-, and 120-min intervals after the introduction of HG. A corresponding negative control (minimal medium with no carbon) was used to monitor changes in O-glycan PUL expression (gray bars). Expression changes were determined relative to the culture grown in PMOG only (time zero [T_0]). (D to H) Experiments similar to those described above, but with levan or chondroitin sulfate (CS) as the competing glycan. Additional experiments for pairwise combinations are shown in Fig. S1 in the supplemental material. (I)

(Continued)

similarity to O-glycans, plus either homogalacturonan or levan to determine whether O-glycan/HMO-responsive PULs are repressed. Both of these substrate pairs elicited clear diauxic growth (see Fig. S4A and S4B in the supplemental material) and repression of known HMO utilization genes when mixed with levan (11) (Fig. 1M).

Expression of some O-glycan utilization functions in other *Bacteroides* species is opposite that in *B. thetaiotaomicron*. In seven other *B. thetaiotaomicron* strains with $\geq 98\%$ 16S rRNA genes identity to the type strain VPI 5482, we observed preferred use of fructans, either levan or inulin (chosen based on which linkage type each strain optimally used), and homogalacturonan when individually mixed with PMOG (see Fig. S4C and S4D in the supplemental material). This commonality among *B. thetaiotaomicron* strains raises the question of whether more distantly related gut symbionts also behave similarly and assign low metabolic priority to O-glycans when they are presented with alternative polysaccharides. Thus, we investigated two other O-glycan-degrading species, *Bacteroides massiliensis* and *Bacteroides fragilis*, with various phylogenetic distances relative to *B. thetaiotaomicron* (Fig. S5A). A survey of the glycans that these two species can use as the sole carbon source revealed that, among the substrates tested, they are restricted to PMOG and starch (*B. massiliensis*), and PMOG, starch, and inulin (*B. fragilis*), respectively (see Table S1 in the supplemental material). Transcriptome sequencing (RNAseq)-based whole-genome transcriptional profiling identified 6 of 33 PULs in *B. massiliensis* and 8/52 PULs in *B. fragilis* that are active on PMOG (Tables S2 and S3). A genome survey revealed that *B. massiliensis* has a PUL with identical organization to the *B. thetaiotaomicron* starch utilization system (*sus*) and is activated on starch (Fig. S5B and S5C). Likewise, *B. fragilis* has two previously described PULs that mediate starch and inulin utilization (28, 29).

Growth of *B. massiliensis* in a mixture of PMOG and starch failed to reveal a distinct diauxic pattern as clear as that observed for *B. thetaiotaomicron*. However, two short pauses in late growth suggested a possible metabolic shift (Fig. 2A) that was supported by individual growth levels on PMOG and starch when tested alone (see Fig. S5F in the supplemental material). Early versus late expression measurements revealed that *B. massiliensis* represses starch utilization until later growth and exhibits neutral or preferred expression of five of the six O-glycan-responsive PULs tested (Fig. 2B). Despite *B. fragilis* exhibiting diauxic growth patterns that appeared to favor either starch or inulin over PMOG (Fig. S5G and S5H), the PULs for these two glycans did not show preferential expression at the growth points analyzed (Fig. 2C and E). In contrast, some O-glycan-responsive PULs were prioritized in early growth, while others remained neutral or were activated later (Fig. 2D and F). The latter finding highlights that independent gene clusters, which otherwise are activated by related glycans from the same source (here, those present in gastric mucin) can exhibit opposing behavior. This observation suggests that

these PULs are individually regulated by opposing signals that vary in their influence on overall expression. These signals likely emanate from both positive and negative cues: recognition of the cognate glycan or repression in the presence of other glycans/sugars, respectively. The contributions of these opposing forces are considered further below.

Differential substrate recognition in complex mixtures contributes to prioritized PUL expression. The above results suggest that simple sugars mediate substantial repression of O-glycan metabolism (Fig. 1K and L), similar to 3'-5' cyclic AMP (cAMP) catabolite repression in *Escherichia coli* and *Bacillus subtilis* (30). However, no evidence for cAMP-responsive pathways has been reported in *Bacteroides* species (31), and we also failed to observe decreased repression of O-glycan-targeting PULs with the addition of cAMP (see Fig. S6A in the supplemental material). We concluded that the presence of polysaccharide surface capsule, which is both phase variable and a known barrier to incoming glycan nutrients (32), is not required for repression of O-glycan PULs under the conditions tested (Fig. S6B). Finally, experiments with a mutant that is unable to utilize levan (*ΩBT1754*) revealed that the mere presence of this preferred glycan in the extracellular environment of a strain that cannot degrade it is insufficient to trigger repression of genes involved in O-glycan use (Fig. S6C and S6D). Collectively, these results lead us to conclude that a cAMP-independent repression mechanism exists in *Bacteroides* that is not passively governed by the presence of bacterial or environmental polysaccharides at the cell surface.

To begin to understand the mechanism(s) underlying *B. thetaiotaomicron*'s prioritization of different glycans, we chose to separate the glycan-sensing capacity of PULs with different responses to O-glycans from their corresponding regulatory elements. To do this, we created two chimeric PULs that were recombined just downstream of their predicted Ton box sequences (Fig. 3A and B; see Fig. S6E in the supplemental material). One of the O-glycan PULs that was chosen for this recombination (genetic locus from *BT4355* to *BT4359* [*BT4355-59*]) was most active during early growth in previous experiments with a partially defined mixture of O-linked glycans and glycosaminoglycans that is termed the "100 mM fraction" (6) based on how it is purified (see Fig. S7A to S7C and the corresponding legend in the supplemental material for details). This same PUL was also weakly and inconsistently repressed in the pairwise glycan mixtures shown in Fig. 1 and Fig. S1. In contrast, the other PUL (*BT4636-31*) showed reciprocal expression during multiphasic growth in the 100 mM fraction (Fig. 3C) and also tended to be more strongly repressed in the pairwise mixtures.

The extracytoplasmic function sigma factor (ECF- σ)/anti- σ factor pairs that regulate these PULs work with outer membrane TonB-dependent transporters via a mechanism termed trans-envelope signaling (33). In this sensory strategy, outer membrane transporters recognize glycans during passage across the outer

Figure Legend Continued

Aggregate expression differences of all PULs involved in metabolizing O-glycans and the non-O-glycans tested. Each symbol shows the value for one separate biological replicate of three conducted for each combination. (J) Exponential growth rates of *B. thetaiotaomicron* on all individual glycans tested. PG, pectic galactan; AP, amylopectin; AG, arabinogalactan; RG I, rhamnogalacturonan I. (Letters over each bar indicate data groupings in which the *t* test *P* values were < 0.001). (K and L) Additional early/late growth phase experiments comparing the responses to a competing polysaccharide (levan and pectic galactan) and their corresponding monosaccharides (fructose and galactose). (M) HMO-responsive PUL (11) expression during early and late growth in a mixture of levan and human milk oligosaccharides (HMOs). A total of three biological replicates were conducted for each experimental condition, including bacterial growth curves. All values are means \pm standard deviations (SD) (error bars), except in panels C and F, where they are means \pm standard errors (SE) (error bars).

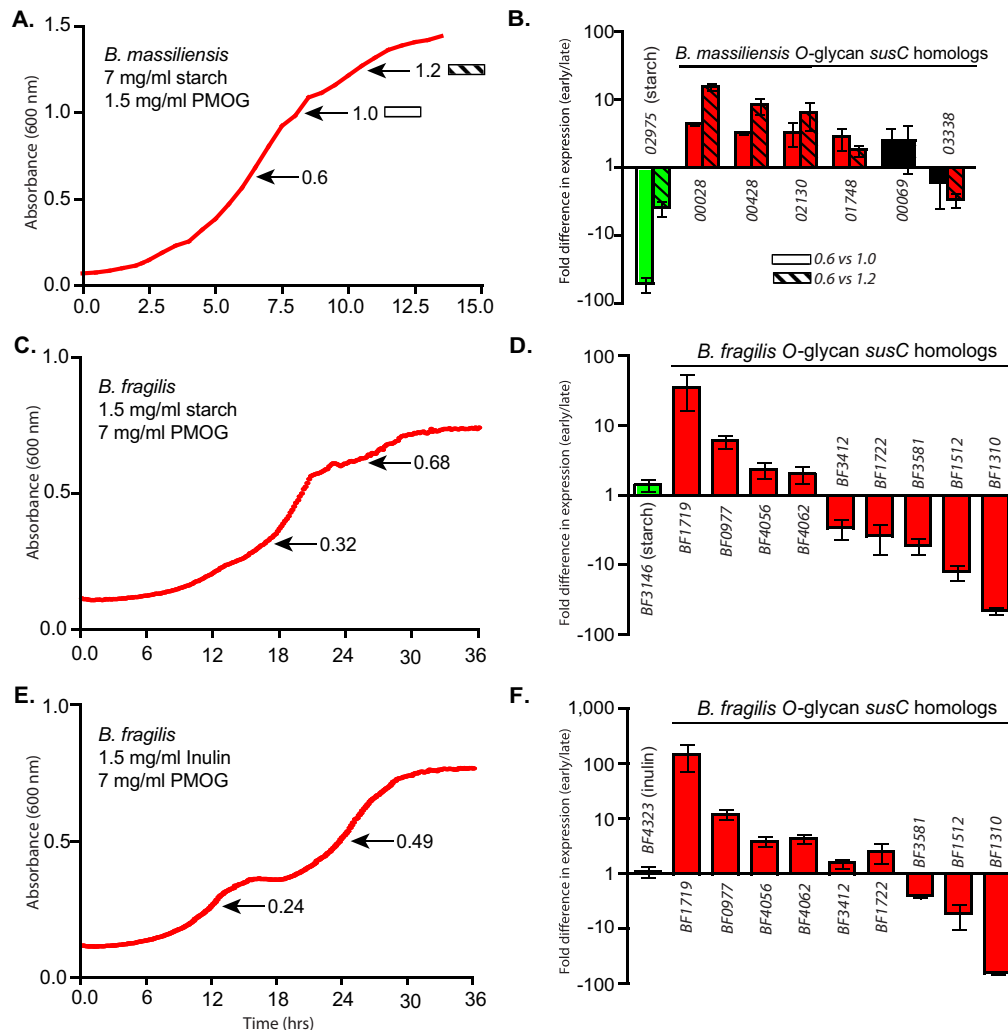


FIG 2 (A) *B. massiliensis* growth curve in a potato amylopectin (starch)/PMOG mix. (B) Relative expression of *B. massiliensis* O-glycan-responsive PULs between early and late growth. Two later growth point transcripts were analyzed (absorbance at 600 nm values of 1.0 and 1.2) and compared separately back to the early growth point ($A_{600} = 0.6$). (C) *B. fragilis* growth curve in a potato amylopectin (starch)/PMOG mix. (D) Relative expression of *B. fragilis* O-glycan-responsive PULs between early and late growth in an APpot (potato amylopectin) (starch)/PMOG mix. (E) *B. fragilis* growth curve in an inulin/PMOG mix. (F) Relative expression of *B. fragilis* O-glycan-responsive PULs between early and late growth in an inulin/PMOG mix. Note the reciprocal amounts of starch and PMOG required for *B. massiliensis* analysis compared to similar experiments with *B. thetaiotaomicron*. All expression values are significant ($P \leq 0.05$ by one-tailed Student's *t* test), except for those values labeled in black. All values are mean \pm SD.

membrane (illustrated as stars in Fig. 3B), and this information is relayed to the corresponding ECF- σ -dependent promoters via protein contacts between the N-terminal domain of the outer membrane transporter, inner membrane anti- σ factor, and cytoplasmic ECF- σ factor (5). Since all of these protein domains are encoded upstream of the Ton box recombination sites, our chimeric PULs were designed to separate the regulatory elements from the downstream regions of the *susC*-like genes that encode glycan-sensing transporter domains (Fig. 3B). Despite their discordant responses in the 100 mM fraction, these PULs are both activated on PMOG and *N*-acetylglucosamine (LacNAc), a major disaccharide building block of O-glycans, as the sole carbon source, suggesting that they respond to similar ligands that may share overlapping structural features (see Fig. S7D in the supplemental material).

Both chimeric PULs were activated during growth on

O-glycans as the sole carbon source, suggesting that chimeric recombination did not eliminate their function (see Fig. S7E in the supplemental material). We then grew wild-type *B. thetaiotaomicron* and a strain that simultaneously contained both chimeric PULs in place of the wild-type loci in the 100 mM fraction that produces discordant phase-specific PUL expression (Fig. 3C). Interestingly, the *susC*-like genes in the chimeric PULs, which were uncoupled from their native regulatory regions, exhibited discordant growth between early and late growth phases that matched the pattern observed for wild-type *B. thetaiotaomicron*. A gene from a control PUL (BT3332), which mediates high-priority chondroitin sulfate catabolism (6), did not show altered phase-specific expression in the chimeric strain. This observation indicates that the regulatory components of these two PULs do not mediate expression differences in this particular case, instead the differential sensing of nutrients via the outer membrane TonB-

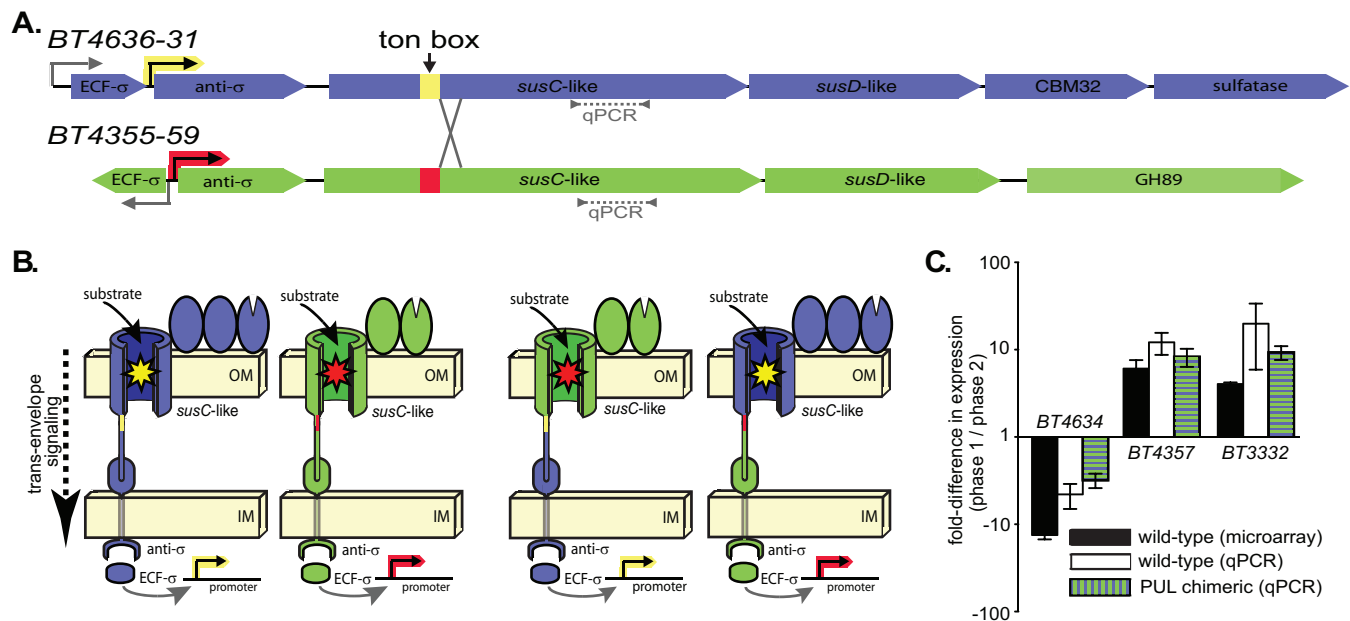


FIG 3 (A) A schematic of the O-glycan-responsive PULs, *BT4636-31* and *BT4355-59*, showing the presumed locations, based on previous microarray data (6), of noninducible promoters (bent arrows), ECF- σ -inducible promoters (colored bent arrows), predicted Ton boxes, and chimeric recombination sites. (B) Cellular organization of chimeric Sus-like systems encoded by the above PULs separating glycan sensing at outer membrane TonB-dependent transporters (yellow and red stars) and genetic regulation via inducible promoters and genes encoding ECF- σ and anti- σ factors. See Fig. S6E in the supplemental material for a detailed illustration of the genetic recombination scheme used. OM, outer membrane; IM, inner membrane. (C) Growth phase-specific expression of *BT4634* and *BT4357* in an undefined mixture containing O-glycans and glycosaminoglycans (GAGs), termed the “100 mM fraction” from PMOG purification (6). Previous microarray (black bars) and current qPCR (white bars and green striped bars) measurements are shown for comparison.

dependent transporter causes this behavior. Despite the fact that these two PULs show responses to similar O-glycan saccharides, such as LacNAc, this behavior could be explained by the sequential release of recognizable saccharides from more-complex structures present in the 100 mM mixture that drive the selective and sequential activation of the two PULs monitored during growth in this mixture. In contrast, if promoter level regulatory events, such as those mediated by cAMP receptor protein (CRP)-cAMP complexes during catabolite repression in other bacteria, predominate in this system, we would have expected growth phase-dependent expression changes to track with each PUL's respective promoter and regulator-encoding genes instead of with *susC*-like sensor/transport regions.

A transcribed intergenic region mediates conditional repression of one O-glycan PUL. In considering additional transcriptional mechanisms that could mediate repression of O-glycan utilization, we noted that six of seven O-glycan PULs that were repressed in Fig. 1 are controlled by ECF- σ factor/anti- σ protein pairs (5), and the genes encoding anti- σ factors and downstream TonB-dependent (SusC-like) receptors are separated by sometimes large intergenic regions (IGRs) ranging from 50 to 197 bp and with no obvious nucleotide homology to each other (see Fig. S11 in the supplemental material for PUL schematics). A previous study in which polar insertions were made in anti- σ coding genes suggested that these genes are cotranscribed (5), and we confirmed this for one PUL (*BT4636-31*) where the genes flanking a 129-bp region could be detected within a single transcript by reverse transcription-PCR (RT-PCR) (Fig. S7F). On the basis of these observations, we hypothesized that these IGRs play a role in O-glycan repression. To test this, we deleted 118 bp (leaving an

11-bp ribosome binding sequence [RBS]) at the 5' end of the cotranscribed IGR in *BT4636-31* PUL that is consistently repressed by alternative glycans (Fig. 4A). Deletion of this region in a mutant strain (Δ *BT4635-34* IGR mutant) had no effect on downstream *susC*-like gene expression when PMOG was the only carbon source, indicating that it does not harbor a required promoter or other sequence required for activation (Fig. 4B). However, when this mutant was grown in either a levan and PMOG mix (Fig. 4C) or pregrown to mid-exponential phase on PMOG and then introduced to levan (Fig. 4D), expression of the downstream *BT4634* transcript escaped normal repression. In fact, it was significantly upregulated during early growth phase in batch culture. Deletion of this particular IGR had no effect on the expression patterns of the six other PMOG PULs (Fig. 4C), indicating that its effect is largely autonomous and restricted to the *cis*-encoded genes that flank it. Despite this discordant PUL expression, both wild-type and Δ *BT4635-34* IGR strains had similar growth profiles on PMOG alone (Fig. S7G). Taken together, these data suggest that the *BT4636-31* O-glycan PUL contains its own *cis*-encoded sequence through which repression is mediated at either the DNA or mRNA level, a phenomenon that may extend to PULs specific for other polysaccharides and in other species.

To determine whether the 129-bp *BT4635-34* IGR was sufficient to cause repression of genes with which it is genomically and transcriptionally associated, we used the Δ *BT4635-34* IGR strain that lacks this sequence in its native PUL as a background and made two genomic constructs in which a *Bacteroides*-adapted *gfp* reporter gene (Fig. 4E) was fused downstream of a constitutively expressed, candidate nonessential (34) *B. theta* gene, *BT0658*. In previous microarray experiments (6, 7, 12), this gene

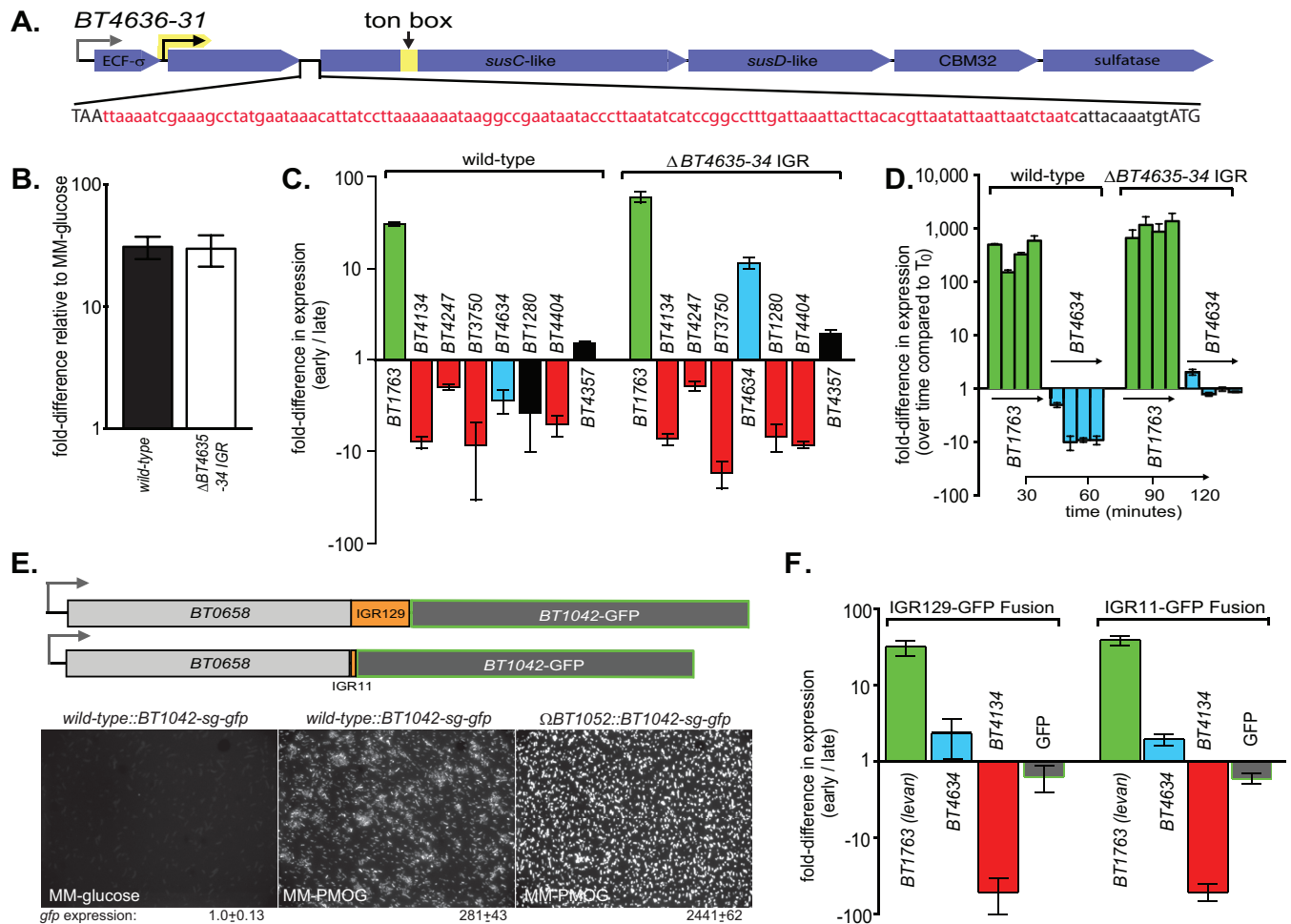


FIG 4 (A) A schematic of the O-glycan-responsive *BT4636-31* PUL showing the IGR deletion sequence. The full IGR sequence is shown in lowercase type with the deleted region shown in red and the retained ribosome binding site (RBS) shown in black. The respective stop (*BT4635*) and start (*BT4634*) codons are in uppercase type. (B) qPCR-based expression analysis of the wild-type and $\Delta BT4635-34$ IGR *B. thetaiotaomicron* strains grown in PMOG as the only carbon source showing full PUL expression in the IGR deletion mutant. (C) Growth phase-specific expression profiles between wild-type and IGR deletion strains in a mixture of levant/PMOG. All other PMOG-responsive PULs previously tested were not altered during IGR deletion. All expression values are significant ($P \leq 0.05$ by one-tailed Student's *t* test) except those labeled in black. (D) Expression analysis from an experiment in which levant was introduced into cells actively growing on PMOG with the same strains shown in panel C and normalized to a no-carbon control as shown in Fig. 1C and F. (E) Fusion of a *Bacteroides*-adapted super-glo *gfp* (*sg-gfp*) reporter gene downstream of the *BT4635-34* IGR sequence and its 118-bp deletion variant and a constitutively expressed gene (*BT0658*) in the $\Delta BT4635-34$ IGR strain as a genetic background. The micrograph images at the bottom validate expression of this reporter. The values below the micrographs are the 16S rRNA gene-normalized expression values by qPCR in MM-glucose and MM-PMOG. The *sg-gfp* gene was adapted for expression in *Bacteroides* by making a translational fusion to the first eight codons of a host glycan-responsive *susC*-like gene, *BT1042*. This gene is expressed in response to PMOG and is also derepressed in a mutant lacking expression of a gene encoding an associated anti- σ factor, *BT1052*. Note that combined loss of *BT1052* and growth in PMOG result in increased *gfp* expression as measured by both qPCR and cell fluorescence. Cells were exposed to oxygen on ice for 30 min prior to imaging to allow the green fluorescent protein (GFP) fluorophore to fold. (F) Relative expression differences between early and late growth phases for the *sg-gfp* reporter strains (with or without the full 129-bp IGR) grown in a mixture of levant/PMOG. Parallel measurements of the levant-responsive *BT1763* gene and the O-glycan-responsive genes, *BT4134* and *BT4634* (here deprioritized in the Δ IGR background) serve as controls.

was expressed, across multiple *in vitro* and *in vivo* conditions, to levels similar to those of genes in the *BT4635-31* transcript during growth on PMOG as the sole carbon source. The two different reporter strains varied only with respect to inclusion of the full IGR between *BT0658* and the downstream *gfp* allele, with one strain containing only the 11-bp RBS sequence that was left behind in the $\Delta BT4635-34$ strain (Fig. 4E). Using this approach, we were able to determine whether the $\Delta BT4635-34$ IGR contained all of the sequence required for orchestrating repression of its native O-glycan-responsive PUL. Results from a batch culture experiment with levant and PMOG as the combined carbon sources

showed similar *gfp* transcript levels between strains, regardless of whether they contained the full IGR sequence (Fig. 4F). As internal controls for the anticipated repressive environment, expression of PUL genes involved in degrading levant (*BT1763*, prioritized) and O-glycans (*BT4634* and *BT4134*) was measured and behaved as expected (note that the background strain in this experiment lacks the 118-bp IGR upstream of *BT4634*, which is why this gene does not show normal repression). Thus, this observation implies that although the 129-bp IGR that lies upstream of *BT4634* is necessary for PUL repression when higher priority nutrients are present, this sequence is not sufficient for this function.

It is therefore likely that sequences contained in the flanking anti- σ and/or *susC*-like genes are required, and future experiments will focus on defining these sequences. Toward this point, it is worth noting that, while we observe conditional repression of the *susC*-like gene (*BT4634*) and other downstream open reading frames (ORFs) in this and other O-glycan PULs, the upstream anti- σ gene (*BT4635*) was not subject to the same growth phase-specific repression (see Fig. S7H in the supplemental material). Provided the insight reported here regarding the role of the intervening IGR in PUL repression, it is probable that this region is the point at which PUL transcript is destabilized by an unknown mechanism and that this event does not equally influence the upstream anti- σ gene.

DISCUSSION

Many bacteria that thrive in complex nutrient environments are capable of utilizing several different substrates. Such microorganisms are often programmed to give preference to certain compounds either because they represent higher energetic value or because they are more easily or consistently accessed. We have previously reported that *B. thetaiotaomicron* exhibits preferential metabolism of some of the >12 different glycans that it can degrade and places O-glycans low in this hierarchy when all are present together (26). Data presented here extend this finding to nearly all single glycan sources accessible to *B. thetaiotaomicron* as well as simple sugars. A possible explanation for this behavior is the cost of degrading the $\sim 10^2$ different glycan structures present on mucins. *B. thetaiotaomicron* activates expression of 86 genes ≥ 10 -fold during growth in PMOG (6). In contrast, activation of only five genes is sufficient (7) to catabolize the simpler and highly prioritized dietary glycan, pectic galactan (Fig. 1L). Indeed, one reason why animals create such diverse O-glycan structures attached to mucins may be to complicate the degradation process of these barrier molecules by microorganisms in order to retain their protective capacity. Given this scenario, it is possible that some bacteria have adapted as specialists for mucin degradation, perhaps by optimizing enzymatic strategies for O-glycan deconstruction or focusing on the dominant, and therefore most economical to harvest, sugars and linkages that are present. *B. massiliensis* and *B. fragilis* appear to be exhibiting a tendency toward specialization for mucin O-glycan degradation, since they utilize only one glycan or two additional glycans, respectively. More strikingly, *B. massiliensis* has even restricted the repertoire of simple sugars that it can metabolize to those found in O-glycans. *B. massiliensis* shows mostly reciprocal preference for O-glycans relative to *B. thetaiotaomicron* when grown on starch, suggesting that it has further optimized its preference for host mucin glycans over dietary substrates.

Collectively, our results provide several new lines of insight into the phenomenon and mechanism of PUL repression in *Bacteroides*. In addition to showing for the first time that individual species can prioritize nutrient mixtures differently, we also delineate part of the pathway through which alternative carbohydrate signals modulate individual PULs and explain variation in PUL expression during growth in mixed nutrients (see Fig. 5 for a working model). On the basis of our experiments with chimeric O-glycan PULs that respond to similar O-glycan cues (Fig. 3), we conclude that substrate sensing is a paramount first step that can vary between PULs in the context of the particular suite of nutrients that are present. This is especially important with mucin

O-glycans that contain many dozen related but variable structures, some of which may harbor critical cues, such as LacNAc disaccharide, within larger chains and are potentially occluded by other surrounding linkages.

When substrate is successfully recognized and degraded, the released monosaccharides, which vary in identity based on the particular glycans present, flow into the cell where they can be sensed by an unknown mechanism, and this information is relayed to individual PULs to modulate their expression. Experiments with one IGR (Fig. 4) imply that this regulatory mechanism requires this region at either the DNA or mRNA level to reduce the abundance of low-priority PUL transcripts. While the complete molecular mechanism through which PUL repression occurs is not elucidated here, it is clear that individual PULs are capable of exhibiting variable responses to different carbohydrates, both with respect to the identity of the alternative glycan (Fig. 1; see Fig. S1 in the supplemental material) or the identity of monosaccharides and timing of the response (Fig. S2). For example, genes within the *BT4636-31* PUL are repressed in the presence of several chemically distinct glycans (levan, chondroitin sulfate, amylopectin, etc.), but not in the presence of heparin, which imposes repression of other PULs in the same bacterial population sampled (Fig. S1A). In contrast, the PUL containing *BT4247* is repressed under the same conditions listed for *BT4636-31*, including heparin, but escapes repression during growth in arabinan, a glycan that imposes strong repression on *BT4634* (Fig. S1H). Deciphering how multiple, chemically distinct signals are integrated into regulation of each individual PUL and also distinguished from one another to create discordant regulatory effects at different PULs will illuminate a key area of global gene regulation in this diverse phylum of important gut symbionts.

Consistent with substrate sensing (i.e., transcriptional activation via glycan cues that may be present but masked by other linkages) being dominant relative to transcription repression in mediating sequential expression of PULs, an additional experiment using the 100 mM fraction as a growth substrate and wild-type and $\Delta BT4635-34$ IGR *B. thetaiotaomicron* strains revealed that growth phase-specific repression of *BT4634* was only partially, yet still significantly ($P = 0.0007$), alleviated with IGR deletion (Fig. 5, inset). Growth phase-specific deployment of the *BT4634* PUL is not governed by the identity of its promoter and regulatory elements, or even its IGR, but instead tracks with the portion of the gene encoding its *SusC*-like transporter and downstream enzymatic functions involved in recognizing an unknown host glycan structure (Fig. 3C). Thus, we conclude that eliminating IGR-mediated repression of this PUL in this particular substrate mixture results in modest loss of repression because its inducing substrate may still not be revealed until later in growth. Prerequisite processing steps (shown as gray sugars attached to the blue inducing saccharide in Fig. 5) may therefore be required to fully activate this system, even in the absence of IGR-mediated repression, and these steps could be conferred by the action of the several additional O-glycan-responsive PULs in *B. thetaiotaomicron*.

Our finding that many different simple sugars exert repression of *B. thetaiotaomicron* O-glycan PULs adds to a previous conclusion by Lynch and Sonnenburg (27), where despite reporting that one monosaccharide (fructose) represses expression of a single PUL involved in arabinan utilization, they concluded that activation of the transcriptional regulator associated with arabinan-

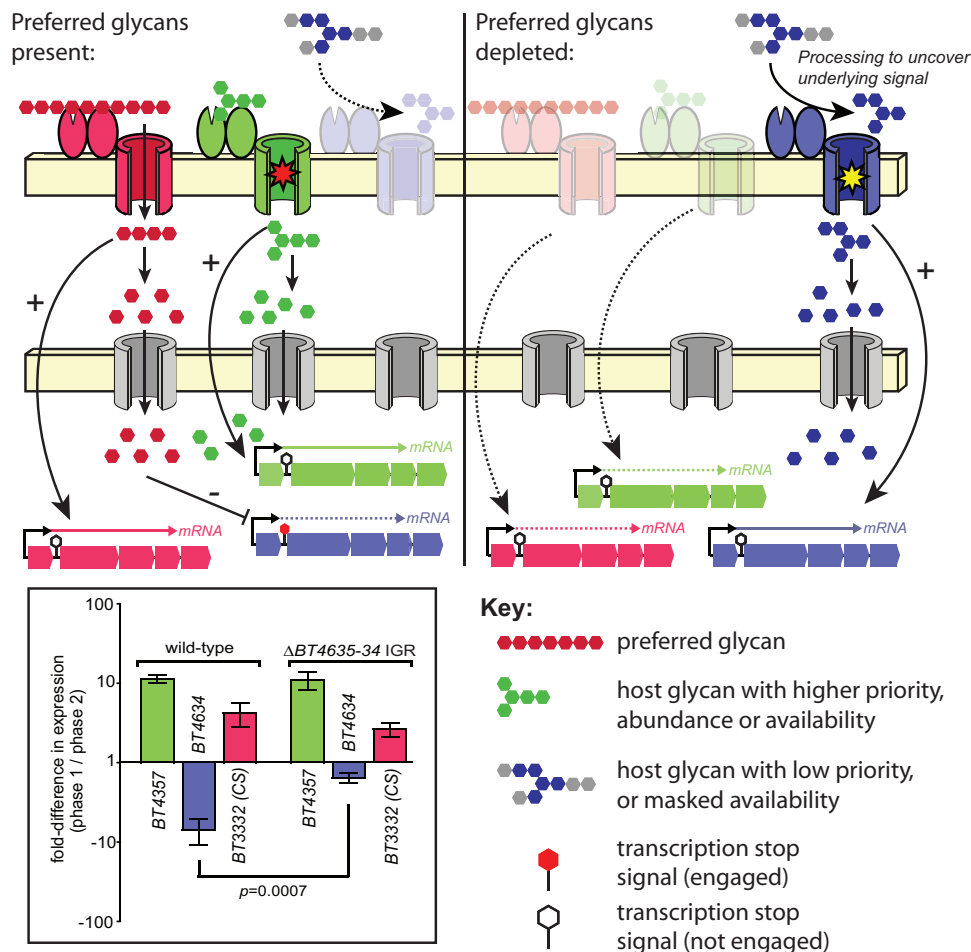


FIG 5 A model to explain the results reported in this study and based particularly on results of expression with the substrate at 100 mM. (Left) In the presence of preferred glycans, such as the CS and heparin glycosaminoglycans, PULs involved in catabolizing high-priority nutrients (red) are activated and liberate simple sugars that are ultimately transported into the cytoplasm, where they mediate repression of PULs that target lower-priority substrates. Since the PULs involved in degradation of some substrates that repress utilization of O-glycans can also be repressed in more-complex nutrient environments (26), it is possible that these gene clusters also harbor elements that mediate transcript repression via either the IGR-mediated mechanism described here or other unknown mechanisms. In the case of the nutrients present in the 100 mM fraction, the inducing substrate for the *BT4355-59* PUL (green) is presumably present in either higher abundance and/or is more accessible, leading to deployment of this system early in growth. Alternatively, systems like *BT4355-59* may be less susceptible to repression by monosaccharides present in certain high-priority substrates, which is consistent with the data shown in Fig. 1 and Fig. S1 in the supplemental material. (Right) After the preferred glycans are depleted and the corresponding repressive sugars are no longer present in the cytoplasm, expression of PULs that target low-priority glycans is released, but may require the action of other enzymes/Sus-like systems to uncover the glycan structure that is recognized by some systems such as that encoded by *BT4636-31* (blue). (Left, inset) Thus, when the *BT4636-31* IGR is absent during growth in the 100 mM substrate, repression is partially relieved (compare blue bars; $P = 0.0007$ by one-tailed Student's *t* test). Since the *BT4636-31* target ligand is sensed later in growth (Fig. 3), possibly because it is only enzymatically uncovered after the action of other systems, this system is not fully deployed early in growth even when its associated IGR is missing.

dependent gene expression is responsible for repression of three genomically unlinked O-glycan PULs (two of which are also monitored here and shown to be repressed). In this case, the natural ligand for the causative transcriptional regulator is known to be arabinan oligosaccharide and not arabinose (7), suggesting that this oligosaccharide cue signals repression of PULs involved in utilizing O-glycans and other substrates. A potential confounder in interpreting these data is that fructose was used via exposure to a chimeric hybrid two-component system regulator that sensed this monosaccharide and activated genes involved in arabinan utilization. While this hybrid regulator was expressed in a *B. thetaiotaomicron* mutant that was impaired for fructose uptake and metabolism, it was still capable of substantial growth on fruc-

tose (28), making it unclear whether the monosaccharide could have also had a direct effect. Nevertheless, these data collectively indicate that multiple mechanisms may be at work to fine-tune global expression of *B. thetaiotaomicron* glycan utilization functions in complex nutrient environments.

Understanding the complex metabolic relationships between dietary and mucosal glycans in the behavior of symbiotic gut bacteria will undoubtedly illuminate new aspects of how the gut microbiota contributes to health and disease. For example, several PULs shown here and in previous work (6) to be repressed by alternative nutrients harbor sulfatase-encoding genes. We have recently shown that sulfatases are required by *B. thetaiotaomicron* to cause inflammation in susceptible mice (24). If knowledge of

diet or prebiotic nutrient conditions can be leveraged to suppress expression of these harmful pathways, it may be possible to reduce or eliminate their contribution to disease states.

Given the vast number of microbial lineages that compose the human gut microbiota and the often large number of different carbohydrate and other nutrient utilization traits encoded in individual species, it is likely that many additional metabolic relationships exist like those described in this report. Indeed, if additional opposing preferences for carbohydrate utilization exist among other gut microorganisms, these behaviors could be a major and unappreciated determinant of how different microbes execute their metabolic potential *in vivo* (35). Understanding these behaviors and their underlying mechanisms will be critical to predicting the behavior and assembly of human gut microbial communities during health and disease and designing therapeutic interventions.

MATERIALS AND METHODS

Growth and genetic manipulation of *Bacteroides*. The type strains *Bacteroides thetaiotaomicron* ATCC 29148 (VPI 5482), *Bacteroides massiliensis* B84634 (DSM17679), and *Bacteroides fragilis* ATCC 25285 (NCTC9343) were used for all experiments except those in Fig. S4 in the supplemental material. Growth profiles and nearly all early/late gene expression experiments were performed using the same conditions and carbon sources as previously described (7), except when noted. *B. thetaiotaomicron* was pregrown in TYG broth overnight at 37°C in an anaerobic chamber (Coy Lab Products) under an 85% N/10% H₂/5% CO₂ atmosphere for all experiments. *B. fragilis* and *B. massiliensis* were pregrown in a custom chopped-meat (CM) broth under the same conditions (36). Prior to any growth or transcript expression experiments, all *B. thetaiotaomicron* and *B. fragilis* strains, including *B. thetaiotaomicron* strains that are not the type strain in Fig. S4, were transferred to minimal medium with glucose (MM-G) for overnight growth. When initially growing *B. massiliensis* in minimal medium, we discovered that it is somewhat fastidious. First, it was unable to grow on glucose alone so we used *N*-acetylglucosamine (GlcNAc) as a carbon source. Second, the addition of the supplements enhanced growth on minimal medium. Thus, we grew this strain in minimal medium containing Balch's vitamins, trace minerals, amino acids, purine/pyrimidines, and beef extract (final concentration of 0.5 mg/ml) also found in our custom CM broth. We then tested for growth on these additional medium components using water as a negative control and GlcNAc as a positive control. We determined that *B. massiliensis* does not have the ability to grow on these substances alone. All strains were then washed in 2× concentration minimal medium with no carbon (MM-NC) prior to conducting any experiment with one source of carbon, glycan mixes, or alternative glycan introductions (spike-in experiments).

All growth curves, except where noted below, were performed in 200-μl cultures in 96-well microtiter plates and a robotic plate stacking and handling device (BioTek) (7). All growth curves were performed at 37°C except that *B. thetaiotaomicron* titration curves from Fig. S1 in the supplemental material were performed at 30°C to help accent the diauxic shift and help determine where harvest points for early and late time points were based on the substrate (three replicate cultures for each mixture ratio; run on separate 96-well plates). However, at 37°C, *B. thetaiotaomicron* behaved similarly when samples were taken for expression profiles. We determined that *B. fragilis* and *B. massiliensis* have limited glycan-degrading capabilities, under the conditions tested, by growing them on a custom glycan array in the same 200-μl culture robotic plate stacking and handling device. All spike-in, RNA-Seq, and 100 mM fraction cell harvests as well as *B. massiliensis* starch/porcine mucin O-glycans (PMOG) growth curves were performed in glass tubes using a UV-visible (UV-Vis) spectrophotometer (Thermo) to monitor absorbance at 600 nm

(A₆₀₀). *B. thetaiotaomicron* mutants and chimeric PUL recombinants were prepared by allelic exchange as previously described (28).

Transcript measurements by qPCR and whole-genome transcriptional profiling by RNAseq. For each glycan mixture tested at early and late time points, three replicate culture sets were assayed. For *B. thetaiotaomicron* and *B. fragilis*, all glycan mixes tested were 1.5 mg/ml non-PMOG and 7 mg/ml PMOG (or human milk oligosaccharides [HMOs]) except for rhamnogalacturonan I (RG I) that was used at 3 mg/ml combined with 7 mg/ml PMOG. For *B. massiliensis*, a mixture of 7 mg/ml amylopectin from potato (APpot) and 1.5 mg/ml PMOG was used for cell harvests. To provide sufficient RNA for analysis, a total of 12 replicate 200-ml cultures were combined into a single 2.4-ml sample representing a series of slightly different harvest points. Thus, the sampling points shown in Fig. S1 in the supplemental material are shown as a distribution of 12 different A₆₀₀ values and their corresponding average. For *B. massiliensis* early and two late glycan-specific PUL transcripts, cells were grown in glass tubes to keep consistent with initial glycan mix growth curves.

To measure spike-in glycan-specific PUL transcripts, *B. thetaiotaomicron* was grown in triplicate under 37°C conditions as described above. Cells were grown on 10 mg/ml minimal medium with PMOG (MM-PMOG) in glass tubes to mid-exponential phase, and the cells were harvested at time zero (T₀). Then, either MM-levan, MM-HG (minimal medium with homogalacturonan), MM-polysaccharide mix (2.5 mg/ml final), or MM-NC was introduced to the MM-PMOG culture. The culture was then allowed to incubate for 2 h while harvesting cells every 30 min. All harvested cells were treated with RNAprotect bacterial reagent (Qiagen) and stored at −80°C until processed. Total RNA was extracted from cells using an RNeasy kit (Qiagen), treated with Turbo DNase I (Ambion), and reverse transcribed using Superscript III (Invitrogen). The 16S rRNA gene-normalized PUL gene abundance was assayed on an Eppendorf Realplex thermocycler and using SYBR FAST premixed qPCR master mix (Kapa Biosystems). All cultures assayed were actively growing on one or more carbon sources, and the use of 16S rRNA gene normalization is rationalized based on extensive comparisons of qPCR measurements with microarray or RNAseq data that were normalized using other algorithms that were not based on 16S rRNA genes (for example, Fig. 3C) (6, 7).

For RNAseq, the total RNA from *B. fragilis* and *B. massiliensis* cells grown on PMOG with glucose and GlcNAc, respectively, used as reference comparisons, was extracted using an RNeasy kit (Qiagen) and treated with Turbo DNase I (Ambion), and finally, rRNA was depleted using the Bacteria Ribo-Zero rRNA removal kit (Epicentre). Residual mRNA was converted to sequencing libraries using TruSeq barcoded adaptors (Illumina) and sequenced at the University of Michigan Sequencing Core in an Illumina HiSeq instrument with 24 samples multiplexed per lane. Bar-coded data were demultiplexed and analyzed using the Arraystar software package with Qseq (DNASTAR).

Illumina RNA sequencing data. Sequencing data were deposited in NCBI under SRA accession numbers SRP035212 and SRP063781.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01282-15/-DCSupplemental>.

Figure S1, PDF file, 1.1 MB.
Figure S2, EPS file, 1.5 MB.
Figure S3, EPS file, 1.4 MB.
Figure S4, PDF file, 0.3 MB.
Figure S5, PDF file, 0.3 MB.
Figure S6, EPS file, 2.3 MB.
Figure S7, EPS file, 2.2 MB.
Table S1, PDF file, 0.1 MB.
Table S2, PDF file, 0.8 MB.
Table S3, PDF file, 0.9 MB.

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